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Original article

Evaluating the Detection Methods for *Arcobacter spp*. Infections in Diarrhea Specimens among Children under Six Years in Arak City

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Abstract

Background: In recent years, the presence of large amount of research isolating and detecting *Arcobacter spp.* from animals and humans with diarrhea and from food samples highlights the importance of *Arcobacter spp.* as emerging food-borne pathogens worldwide. Recently, independent studies have been conducted, making significant progress in the understanding of the classification and pathogenicity of this group of microorganisms. However, the incidence of *Arcobacter* infection is likely to be underestimated mainly due to the limitations in current detection and identification methods. This study was done to evaluate the effectiveness of staining method (Gram stain using 1% fuchsine in direct smear) versus PCR as the gold standard.

Materials and Methods: A total of 150 stool samples were collected from under 6 years of age children with diarrhea from clinical centers of Arak. Direct smears of samples were stained with a modified gram staining method (1% fuchsine for 5 minutes with heat). Concurrently, PCR amplification method was performed for all DNA samples.

Results: *Arcobacter spp.* was isolated by PCR from 28 out of 150 stool samples. Direct staining method identified 79 samples as *Campylobacter*-like organisms with a sensitivity and specificity values of 100 and 65.50%, respectively.

Conclusion: Detection of *Campylobacter*-like organisms by 1% fuchsine is simple, inexpensive, and fast with high sensitivity and specificity. Laboratories with limited resources can employ modified gram staining method to detect *Campylobacteriaceae* infection in early stages.

Key words: Arcobacter, Direct observation, PCR, Diarrhea

1. Background

The genus *Arcobacter* has been classified together with the genus *Campylobacter* into the family *Campylobacteraceae*. They are aerotolerant, *Campylobacter*-like organisms, previously classified as *Campylobacter. Arcobacters* are rod, gram negative, non-spore forming, motile, curved, and occasionally straight bacteria(1). In direct smear, it can be seen in *Campylobacter*-like bacteria. *Arcobacters* are helical rods of 1-3 µ by 0.2-0.4 µ, and sometimes may produce long cells up to 20 µ. They have single polar flagellum and display typical corkscrew-like motility, and in old culture, morphology of cells changes from spiral to coccoid forms. (2, 3). Some *Arcobacter* species have been isolated from stool samples of patients with and without diarrhea and occasionally in association with bacteremia, endocarditis, and peritonitis (4-8). In this regard, animal products are considered as important routes for the *Arcobacter spp*. transfer.

Arcobacter spp. is a prevalent contaminant of broiler carcasses in time of poultry processing, and contaminated poultry products are the most significant sources of *Arcobacter spp*. infection for humans (9, 10). There is increasing evidence showing that livestock animals are significant reservoirs of *Arcobacter spp*. worldwide. Over the last few years, the presence of these organisms in animal products such as raw meat has received increasing attention (cattle, poultry, pigs) (11, 12). Contamination of fresh vegetables such as lettuces and spinach with *Arcobacter spp.* has also been reported only recently. Given that these foods are generally considered as safe and consumed in large quantities and the fact that further cooking is absent, vegetables could be considered as a source of *Arcobacter spp*. and a public health concern (13). Many studies have shown that *Arcobacter spp*. has been detected in various types of water including bays, ground water, surface water, raw sewage, and sea water. These contaminated water sources could act as a carrier for the *Arcobacter spp.* transfer to humans and animals (14-18). In addition to consuming animal products and drinking water, direct contact with infected humans or animals is another potential source of *Arcobacter spp.* transfer (19-21). The majority of *Arcobacter spp*. isolated from different animals belong to three species: *A. butzleri, A. cryaerophilus*, and *A. skirrowii. A. butzleri* has been isolated frequently from humans with diarrhea. *A. butzleri* shows microbiological or clinical characteristics very similar to *Campylobacter jejuni*. But persistent and watery diarrhea is the main symptom associated with *A. butzleri* in contrast to the bloody diarrhea found in *C. jejuni* infections (22). The common symptoms of *Arcobacter* infection are persistent diarrhea accompanied by abdominal pain, stomach cramps, and symptoms such as nausea, vomiting, and fever and at times, symptoms are so severe that hospitalization may be required (21-23). Despite a range of isolation methods used, no standard method for the isolation of *Arcobacter spp*. from fecal samples has been established. Many of the suggested protocols are time-consuming and expensive for the isolation of this bacterium. The most common method for isolating *Arcobacter spp.* and other *Campylobacter*-like bacteria from human's clinical specimens is a combination of enrichment–filtration and selective agar in parallel (24). This method is time-consuming and sometimes associated with a high rate of fecal contamination, causing the reading of plates to be

arduous and the time to be waste. Therefore, most laboratories in developing countries do not routinely perform tests to detect *Campylobacter*-like organisms. Access to cheap, sensitive, and specific methods would assist in detecting *campylobacter*-like bacteria and their epidemiology. Alternate methods such as gram staining of direct smear have a sensitivity of 60-90% and a specificity of 99.5% for the detection of *Campylobacter* species directly from stool samples (25, 26). The present study examined the sensitivity and effectiveness of the staining method for *Arcobacter* species.

2. Objectives

The aims of this study were to isolate *Arcobacter spp.* from the stool samples of patients with gastroenteritis and to evaluate the effectiveness of the staining method (Gram stain using 1% fuchsin direct stain) versus PCR as the gold standard.

3. Materials and methods

3.1. Sample collection

In this descriptive cross-sectional study, infectious diarrheal stool specimens were collected from 150 children referred to the educational and medical centers affiliated with the Arak University of Medical Sciences (Amir Kabir) during July to September 2016. None of the children had taken antibiotics for at least one week before entering the study. The study questionnaire was completed after obtaining written consent form from the patients or their parents or guardians.

3.2. Method

At first, a thin smear was prepared from the mucous area of feces. Smear was allowed to air dry and subsequently fixed by methanol, and then the smears were stained with modified gram staining method. Staining was performed by covering the smears with 1% fuchsine as contrasting color for 5 minutes, the smears were heated until vapor just begins to rise. All slides were observed under light microscope using 10x magnification for white blood cells and 100 x oil immersion magnifications for morphological appearance of *Campylobacter*-like organisms.

Concurrently, DNA was extracted from all samples, followed by PCR. The genomic DNA was extracted from the entire 150 stool samples using the Stool DNA Isolation Mini Kit (YTA, Iran) as described by the manufacturer. The concentration of each DNA was determined spectrophotometrically at 260 and 280 nm. Adjusted to 20 $mg.\mu L^{-1}$, extracted DNA samples were stored at –20 °C until the PCR analysis. PCR assay was done on DNA extracted from stool samples directly. For the genus-specific PCR, the primers Arc1 and Arc2 targeting a section of the *16SrRNA* gene were used. PCR amplifications were performed in a final volume of 25 µL consisted of 3 µL of the DNA template, 12.5 µL PCR Master Mix Red (1.5 mM $MgCl_2$; Ampliqon, Denmark), and 0.7 µL (10 pmol) from the forward and reverse primers (CinnaGen, Iran) (Table 1). The volumes of the reaction mixtures were reached 25 mL using sterile water (molecular grade).

The PCR cycling was performed in a gradient thermal cycler and set under the following conditions: an initial denaturation at 94°C for 5 min, followed by 28 cycles of denaturation at 94°C for one min, annealing at 52.7°C for 55 s, extensions at 72°C for 55 s, and a final extension at 72°C for 8 min. PCR products were separated by electrophoresis on 1.5% agarose gel and stained with 1% safe stain (CinnaClon, Iran). A 100-bp DNA ladder was used as a molecular size marker. The bands were visualized and recorded in gel documentation system Quantum ST4 (Vilber Lourmat, Eberhardzell, Germany). The DNA of the reference strains *A. butzleri* ATCC 49 616 was used as positive control, and sterile water (molecular grade) was used as a negative control in PCR experiments.

4. Results

4.1. Clinical symptom

A total of 150 children's diarrheal stool samples were collected from clinical centers in Arak. Of which 49% were watery, and 51% were mucoid. Samples were collected from febrile persons (66%), persons with abdominal pain (88.75%), vomiting (42.5%), and nausea (64%).

4.2. Results of direct microscopic observation (staining)

Using staining, of 150 collected samples, 79 samples (52.66%) showed the morphology compatible with *Campylobacter*-like organisms (*Campylobacter spp*, *Helicobacter spp*, and *Arcobacter spp*). Bacteria were observed in smear as gram negative rods in spiral and gull-winged forms. The bacterium with these characteristics is thought to be the *Campylobacter*-like organism (Figure 1).

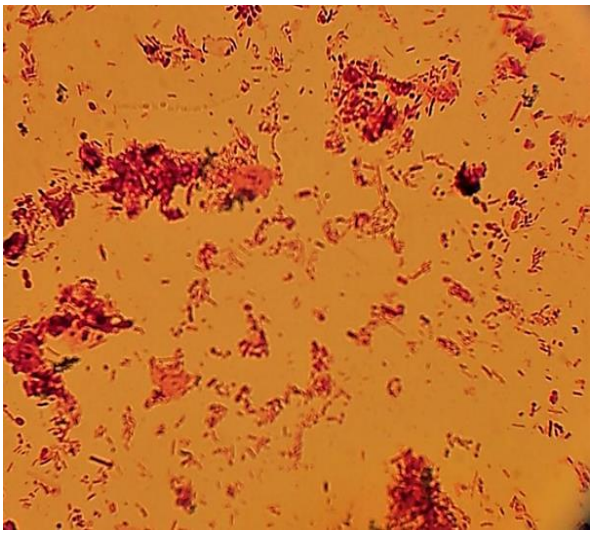

Figure 1. Modified Gram staining of stool sample with 1% fuchsine (*Campylobacter* like organisms: gull-winged shaped, Gram-negative rods).

Table 1. Nucleotide sequences used as primers in the PCR reaction for identification of *Arcobacter* genus.

Name of Primer	Sequence (5` to 3`)	Target Gene	Product Size (bp)	Reference
Arc 1 Arc 2	AGAACGGGTTATAGCTTGCTAT GATACAATACAGGCTAATCTCT	*16SrRNA*	181	(Gonzalez *et al.* 1999)

4.3. Genus Level Identification of Arcobacter from Clinical Specimens PCR

Of 150 samples examined, 28 samples (18.66%) were identified by PCR as *Arcobacter* genus (Figure 2).

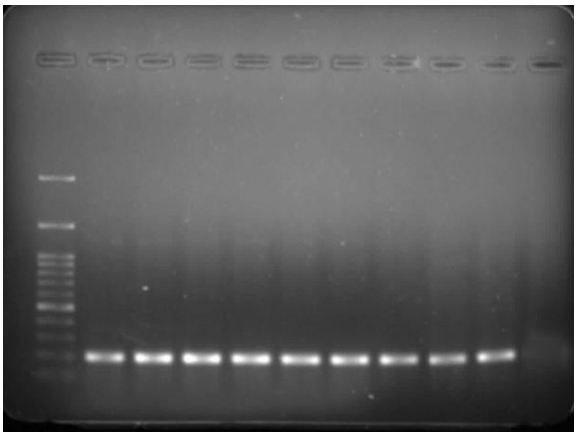

Figure2. Identification of *Arcobacter* isolates at the genus level by PCR. Lanes: 1, 100 bp size marker; 2: positive control *A. butzleri* ATCC 49616. 3–10, positive samples; 11, negative control (sterile water (molecular grade)).

Table 2. Microscopic (1% fuchsine as the opposite color) versus PCR as reference standard.

Molecular	Microscopic		Total
	+	-	
+	28	0	28
-	51	71	122
total	79	71	150

Sensitivity and specificity of diagnostic methods based on PCR method was calculated using MedCalc statistical software , the results are shown in Table 3.

Table 3. Sensitivity and specificity of the microscopic method compared to PCR.

Method	Number of Positive Samples	Sensitivity	Specificity
Direct microscopic observation	79	100%	65.50%

The association between the presence of white blood cells in infectious stool and the presence of *Arcobacter* species was investigated (Table 4). Of 51 stool specimens with WBC, 35 cases had *Arcobacter spp*, and of 99 stool specimens with no WBC, only 44 had *Arcobacter spp*. infection.

Table 4. Relationship between the presence of WBC and *Arcobacter* species in direct smear of feces.

Total	Presence of *Arcobacter spp*. in Direct Smear		WBC
	+	-	
51	35	16	+
99	44	55	-
150	79	71	Total

5. Discussion

Gram staining is used as the principle staining method in most laboratories of developing countries and as a critical step in the diagnosis of bacterial infections. Its efficiency for the detection of *Campylobacter* species using 0.3% carbolfuchsin has been documented.

In the current study, in direct gram smear of 150 samples, 79 (66.62%) samples were positive. Gram staining for the detection of *Arcobacter* specie in stool samples had a sensitivity of 100% and a specificity of 65.50%. But one must be careful in not reporting all of the 79 cases as *Arcobacter spp*. since gram negative rods in spiral and comma form and gull-winged shape observed in smear are compatible with all of the *Campylobacter*-like organisms (including *Campylobacter spp*., *Arcobacter spp*. and *Helicobacter spp*.).

Several studies detecting *Campylobacter spp*. with direct observation of clinical cases have been reported. Martha Fidelis Mushi et al. (2013) in Tanzania compared the staining methods versus culture as the gold standard for diagnosis of *Campylobacter*. Of 300 tested specimens in culture, 14 cases were positive, and 28 positive cases were stained (27).

In another study in India in 2014, in order to compare the 3 methods of direct observation, culture, and PCR, all of which were performed to detect *Campylobacter spp*. The sensitivity of the PCR method as the gold standard was 96.69%, the culture was 37.19%, and the direct examination was 63.64%. It was reported that the direct smear method is better than the culture method in the selected medium (28).

In a study in Chile in 2016, the sensitivity and specificity of the staining method for the detection of *Campylobacter spp.* was reported to be 6.5 and 100%, respectively (29). Another study conducted in the same country in 1982 showed a sensitivity of 43.5% and a specificity of 99.4% for this diagnostic method.

In New Zealand in 2004, the sensitivity and specificity of the direct microscopic examination method for the detection of *Campylobacter spp.* in the stool samples taken within 30 minutes after sampling were reported to be 89 and 99.7%, respectively (26)

In 2010, a study was performed to specify the sensitivity and specificity of Gram stain of the stool in diagnosing *campylobacter* infection using culture as the gold standard. The sensitivity and specificity of warm staining in *Campylobacter spp.* detection were reported to be 76 and 99.5% in Charcoal Cefoperazone Deoxycholate Agar (CCDA medium) (24).

The main symptoms of *Arcobacter* infections include abdominal pain, nausea, vomiting, and fever. In a study in Turkey, the most common symptoms were nausea, abdominal pain, and fever (30). In another study in France, severe diarrhea, abdominal pain, and fever have been reported as the symptoms of the disease (31).

In an *A. butzleri* outbreak affecting 10 children in an Italian school, the main symptom was recurrent abdominal cramps without diarrhea; the infection was so severe that requires the hospitalization of the 3 children (32).

In the current study, the most common symptoms were similar to the results reported by other studies, including abdominal pain, diarrhea, nausea, and sometimes fever.

Out of 79 positive samples in direct gram smear, 28 specimens were confirmed by PCR as genus *Arcobacter*. The most common symptoms among these 28 specimens were abdominal pain, fever, nausea, and more watery diarrhea (65.9%) with fewer white blood cells (18.43%).

On the other hand, common symptoms were mucoid diarrhea, bloody diarrhea, and high levels of WBC, which is similar to the infection caused by *Campylobacter spp*. According to another study carried out simultaneously, out of

160 samples tested the prevalence rate of *Campylobacter spp.* was reported as 92 and 74 cases in direct gram smear and in molecular method, respectively (unpublished study).

Based on the results of this study and several other studies, it was determined that *Arcobacter spp*. causes mainly watery diarrhea, while *Campylobacter spp.* causes more mucoid diarrhea and less watery diarrhea. It was also found that *Arcobacter spp.* rarely causes bloody diarrhea, while *Campylobacter spp.* can cause bloody diarrhea.

In the current study, the presence of white blood cells in stool was detected in small number of positive samples. This finding has been confirmed in several other studies (22, 30). Although the presence of leukocyte was significantly associated with *campylobacter* infections, in previous studies the occurrence of white blood cells in stool has been reported in 25-90.4% of the culture positive cases of *Campylobacter* species infections (25, 33). It should also be noted that *Arcobacter*-induced diarrhea is more persistent than *Campylobacter* diarrhea.

According to the symptoms reported from other *Campylobacter* like organisms, the remainders of the positive samples by smear could belong to the other genera of the *Campylobacteraceae* family. It should be noted that the direct gram stain examination is highly dependent to the technician skill and the type of staining, so it is very difficult to evaluate this method, and as said before, the results should be reported as *Campylobacter*-like organisms, including *Arcobacter*, *Campylobacter*, and *Helicobacter*.

Laboratories with limited resources for culture or molecular methods with regard to the clinical symptoms in areas where *Campylobacter*-like organisms are prevalent, could adopt this method as a routine method, especially during high incidence seasons.

6. Conclusion

Although the Gram staining method requires high skilled experienced microscopists, it can be considered as a simple technique for providing presumptive results in a short time with relatively high sensitivity and low cost.

Conflicts of interest

The authors declare they have no conflict of interests.

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Authors' Contributions

All authors contributed equally in this research.

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